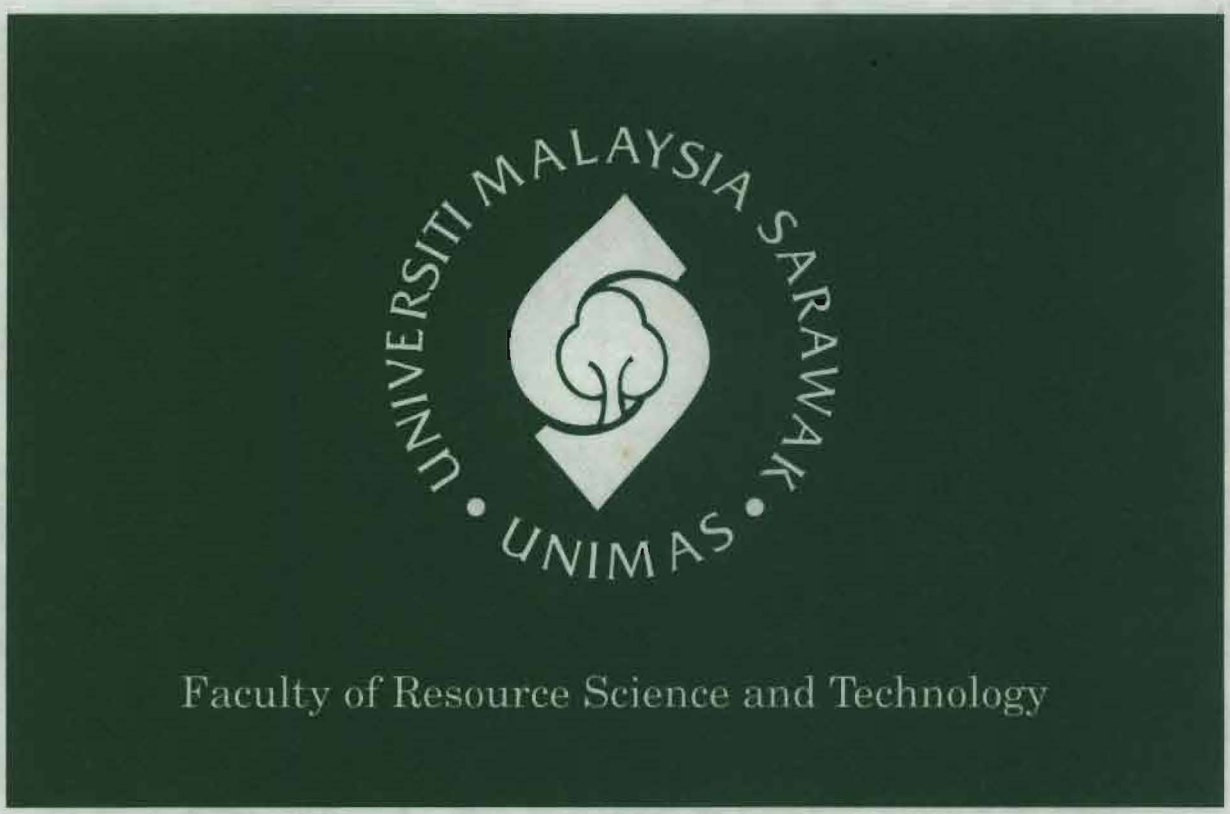

IDENTIFICATION OF GENETIC MARKER IN FIVE SELECTED FAMILIES IN SUBORDER MICROCHIROPTERA USING PCR-RFLP OF CYTOCHROME OXIDASE I GENE

Wan Rusilawati Binti Wan Rahin

Bachelor of Science with Honours
(Animal Resource Science and Management)
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WAN RUSILAWATI BINTI WAN RAHIN

This project is submitted in partial fulfillment of the requirements for the degree of
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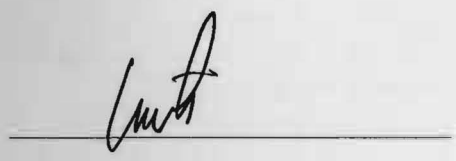
(Animal Resource Science and Management Programme)

Faculty of Resource Science and Technology
UNIVERSITI MALAYSIA SARAWAK
2006

DECLARATION

No portion of the work referred to in this dissertation has been submitted in support of an application for another degree of qualification of this or any other university or institution of higher learning.



Wan Rusilawati Binti Wan Rahin

Animal Resource Science and Management Programme
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak

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ABSTRACT

PCR-RFLP of mitochondrial cytochrome oxidase I (COI) gene was used as a genetic marker to differentiate five selected families (Emballonuridae, Megadermatidae, Nycteridae, Rhinolophidae and Hipposideridae) of suborder Microchiroptera. Twenty four species were used to represent the families. Of ten restriction enzymes used, only six (*Rsa*I, *Csp6*I, *Alu*I, *Sal*I, *Hpa*II and *Hae*III) showed polymorphism but there were no specific site showing family relationship of all the species used. However, only *Csp6*I is diagnostic to differentiate some species from the genus *Rhinolophus* and *Hipposideros*. This study showed that PCR-RFLP technique is a good genetic marker.

Key words: Genetic marker, cytochrome oxidase I (COI), PCR-RFLP.

ABSTRAK

*PCR-RFLP dari gen mitokondria cytochrome oxidase I (COI) digunakan sebagai penanda genetik untuk membezakan lima famili (Emballonuridae, Megadermatidae, Nycteridae, Rhinolophidae dan Hipposideridae) suborder Mikrokiroptera terpilih. Dua puluh empat spesies digunakan bagi mewakili famili-famili tersebut. Dari sepuluh enzim pembatasan yang digunakan,hanya enam (Rsa*I, *Csp6*I, *Alu*I, *Sal*I, *Hpa*II dan *Hae*III) *yang menunjukkan perbezaan tetapi tiada yang menunjukkan tempat spesifik yang menunjukkan hubungan kekeluargaan antara semua spesies yang digunakan. Walau bagaimana pun, hanya Csp6*I *yang boleh membezakan sesetengah spesis dalam genus Rhinolophus dan Hipposideros. Kajian ini menunjukkan bahawa teknik PCR-RFLP ialah penanda genetik yang baik.*

Kata kunci: Penanda genetik, cytochrome oxidase I (COI), PCR-RFLP.

1.0 INTRODUCTION

Chiroptera is the second largest order of mammals with 188 genera and 977 species in the world (Corbet and Hill, 1992). The unique feature of this order compared to other mammals is that they are capable of true flight like birds (Vaughan, 1986). Bats are widely dispersed throughout most of the world because of their ability to fly (Ditchfield, 2000). The modification of forelimbs to wings is believed to have evolved from some type of insect-eating mammal (Richardson, 1985; Martin *et al.*, 2001). Chiroptera is divided into two distinct suborders, Megachiroptera and Microchiroptera. Megachiroptera consists of a single family, Pteropodidae while all the other families are microchiropterans (Payne *et al.*, 1985).

Microchiroptera consists of 147 genera and 814 species (Corbet and Hill, 1992). Most Microchiroptera have tragus or antitragus. They prefer to roost in enclosed sites (Corbet and Hill, 1992). Insectivorous bats have the ability to locate their prey by using echolocation which are ultrasonic pulses produced by the larynx (Vaughan, 1986). This call is inaudible to human's ears and species specific (Fenton, 1997). Since echolocation is used in maneuvering and foraging instead of vision, microchiropteran eyes do not develop well, but their nose and facial morphology are often evident (Feldhamer *et al.*, 1999). Their food habit made them useful as an insect controller. Gumal *et al.* (1998) stated that insectivores consume large amount of insects per night. For instance, 1.8 million wrinkle-lipped bats (*Tadarida plicata*) at Mulu National Park eat approximately 9 tonnes of insects per night (Gumal *et al.*, 1998).

There are seven families of Microchiroptera in Borneo, namely, Emballonuridae, Megadermatidae, Nycteridae, Rhinolophidae, Hipposideridae, Vespertilionidae and Molossidae (Payne *et al.*, 1985). Among those, only five families were studied in this research that is Emballonuridae, Megadermatidae, Nycteridae, Rhinolophidae and Hipposideridae. Rhinolophidae and Hipposideridae were chosen because according to Corbet and Hill (1992), they were classified as one family. The other three families were also chosen as an additional since the research was carried out to study family relationships.

Mitochondrial DNA (mtDNA) encodes gene products required for mitochondrial protein synthesis, electron transport and oxidative phosphorylation (Garrett and Grisham, 2002). Mitochondrial DNA is widely used in population genetics and evolutionary study because of its maternal and non-recombining mode of inheritance; its rapid evolution and its intraspecific polymorphism (Hartl and Jones, 2001; Szalanski and Owens, 2003). It is used to understand evolutionary relationships among individuals, populations and species (Irwin *et al.*, 1991). Vertebrate mtDNA have a control region, 13 genes, 20 tRNAs and 2 rRNAs. It is a closed circular DNA with approximately 17,000 base pair (bp) (Avise, 1994).

Cytochrome oxidase *I* (COI) is also called cytochrome c oxidase because it accepts electron from cytochrome c and directs them to the four electron reduction of O_2 to form H_2O (Figure 1). This gene consists of 13 subunits, and it contains two heme centers (heme *a* and a_3) as well as copper atom (Garrett and Grisham, 2002). According to Herbeck and Novembre (2003), the length of COI gene fragment is approximately 1.5 kb. Due to the large size and is highly conserved, COI is

useful in studying molecular phylogenetic (Lunt *et al.*, 1996). COI gene evolves more slowly than other protein coding genes in mitochondria, but it has rapid mutation rate (Simon *et al.*, 1994).

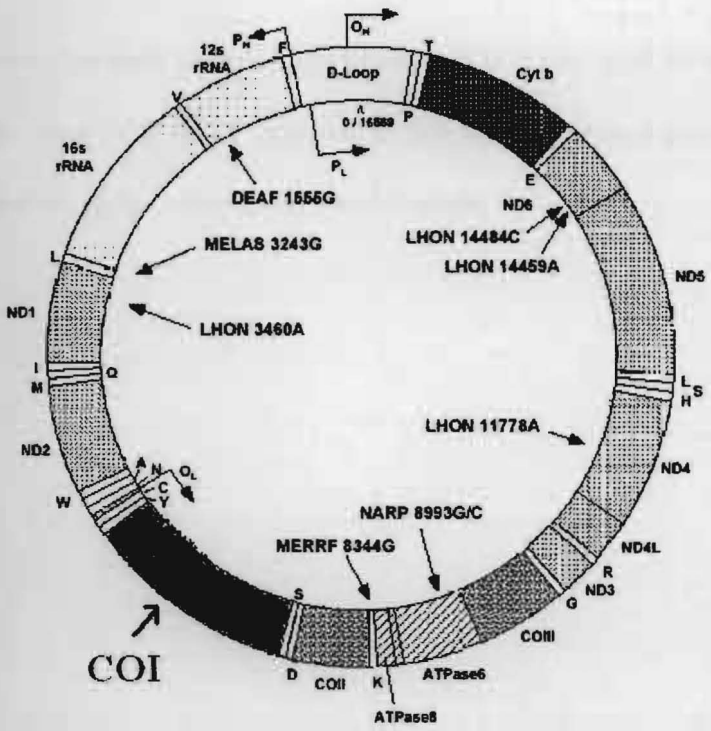

Figure 1: Gene map of the mitochondrial DNA and the location of COI. (Source: Anon, 2002)

Restriction Fragment Length Polymorphisms (RFLP) is a cheaper method to study molecular genetics rather than DNA sequencing. This technique is useful to analyze a variety of genetic systems. RFLP analyses of mtDNA are marginally informative for the study of genetic identity, parentage and closely related species; and highly informative to study conspecific population (Avise, 1994). This technique uses restriction endonucleases (enzymes) to cut DNA at a specific recognition sequence (restriction site), usually four to six nucleotides pairs in length. The

restriction enzymes are produced by different species and strains of bacteria (Fincham, 1994). One more advantage of this technique is that the PCR product does not need to be purified (Levy *et al.*, 2002).

COI gene was chosen to be used in this study because it is rarely used in studying vertebrates. This study was done using PCR-RFLP analysis to test whether it is a good genetic marker in resolving selected families in the suborder Microchiroptera.

2.0 LITERATURE REVIEW

2.1 Cytochrome oxidase I (COI)

Nylander *et al.* (1999) used COI to test the monophyly of the gutless annelid group within the Phallodrilinae using mtDNA sequence data. The study was carried out because there is no complete phylogeny of the gutless species and their position within the Phallodrilinae is unclear. Furthermore, previous phylogenies were constructed based on morphological data. The study showed that the COI gene fragment supports monophyly of the gutless group within the Phallodrilinae (Nylander *et al.*, 1999).

Arévalo *et al.* (2004) found out that the use of COI as genetic marker has improved the resolution of Polistinae (Hymenoptera) phylogenetic tree. The research showed that the combination use of appropriate genetic marker and molecular tool could generate a good phylogenetic tree.

Vandewoestijne *et al.* (2004) used mtDNA to clarify the phylogenetic relationships within and between several *Aglais urticae* (Lepidoptera) subspecies. Two regions (control region and COI) were amplified and sequenced to produce minimum spanning networks data and maximum likelihood tree. The results showed that control region have less genetic variability than COI gene.

The phylogenetic position of Aeolosomatidae and Potamodrilidae within annelida is unclear. Struck and Purschke (2005) used COI and 18S rDNA to resolve the problem. The results showed that Aeolosomatidae and Potamodrilidae are sister group.

2.2 Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR- RFLP)

Lovette *et al.* (1999) used three molecular techniques (RFLP, DNA sequencing and PCR-RFLP) to characterize the maternal ancestry of hybrids of four Pacific North-west bird species (*Dendroica townsendi, D. occidentalis, D. virens and D. nigrescens*). PCR-RFLP assay produce species-specific haplotypes in the 'pure' *D. townsendi* and *D. occidentalis* populations and the co-occurrence of those haplotypes in the hybrid population. From RFLP and sequence data, they found that the differences within *D. nigrescens* are greater than the difference between *D. townsendi* and *D. occidentalis*. *D. virens* and the ancestor of *D. townsendi* and *D. occidentalis* split one million years ago; *D. townsendi, D. occidentalis* and the populations of *D. nigrescens* split about 400,000 years ago.

A study by Rohwer *et al.* (2001) used the same molecular marker as in Lovette *et al.* (1999) to study the hybrid zones of two species of warblers (*D. townsendi and D. occidentalis*) in Washington. Using the three methods the research concluded that there is contrasting in association between phenotypes and mtDNA haplotypes suggest that *D. townsendi* are invading the Washington hybrid zones from the north.

Vidigal *et al.* (2002) used PCR-RFLP to differentiate Brazilian *Biomphalaria* snail species using COI region, digested with *Alu*I and *Rsa*I. The study involved three species (*Biomphalaria glabrata, B. tenagophila* and *B. straminea*) and there were also representatives of each species from different populations. Digestion with *Alu*I produced interspecific and intraspecific variation, while *Rsa*I only produce interspecific variation.

Meagher and Gallo-Meagher (2003) used two molecular techniques (mtDNA RFLP and PCR-RFLP) to identify host strains of fall armyworm (*Spodoptera frugiperda*). Two strains of fall armyworm were identified (corn strain and rice strain) and both strain are morphologically similar. MtDNA RFLP was carried out by using double digestion (*Hae*III and *Msp*I) while restriction enzyme used for PCR-RFLP was *Msp*I. Result showed that both techniques can differentiate the strains but PCR-RFLP can produce more accurate polymorphic site.

Ferreira *et al.* (2005) used PCR-RFLP method to discriminate between two closely related of bat species, namely *Platyrrhinus lineatus and P. recifinus*. These two species had similar morphological characteristics. Seven studies had been carried out using classical method but none of them can pin point the exact features that make these two species distinct. So, the study was conducted using molecular method (PCR-RFLP) and they found that *P. lineatus and P. recifinus* are two distinct species.

Since the fish juveniles of winter skate (*Leucoraja ocellata*) and little skate (*L. erinacea*) are difficult to be identified, Alvarado Bremer *et al.* (2005) used PCR-RFLP method to solve the problem. The amplified segment of COI was digested with *Sty*I. The study produced genetic markers which successfully identifying the cryptic juveniles of the skate species.

3.0 MATERIALS AND METHODS

3.1 Tissue collection

Fresh samples were collected from various localities in Sarawak namely Bako National Park, Niah National Park, Kubah National Park, Similajau National Park, Matang Wildlife Centre, Bau limestone area and Sematan. Preserved samples from various localities that are available at the Faculty of Resource Science and Technology Zoological Museum, Universiti Malaysia Sarawak (UNIMAS) were also used (Appendix 1).

3.2 DNA extraction

DNA samples were extracted from the muscle tissue of 25 selected species from the five families. Extraction from tissue sample was done by using C-TAB method (Grewe *et al.*, 1993). About 0.1 to 0.2 g tissue sample was grounded in a 1.5 ml microcentrifuge tube with 700µl of 2×CTAB buffer and 5µl of Proteinase K (20mg). Then, the sample was incubated at 60°C until completely dissolved. Seven hundred micro-litre chloroform-isoamyl alcohol (24:1) was added and the tube containing the mixture was centrifuged at 13,000 rounds per minute (rpm) for 10 minutes. The upper aqueous phase containing DNA (550µl) was transferred to a new tube, and 550µl of 100% ethanol (EtOH) was added. The sample was centrifuged at 13,000 rpm for 10 minutes. All liquid from the microcentrifuge tube was removed and 600µl of cold 70% EtOH and 25µl of 3 M NaCl

was added. The mixture was then centrifuged at 13,000 rpm for 10 minutes. All liquid from the microcentrifuge tube was removed and the pellet was air dried. Lastly, the pellet was dissolved in 50µl of sterilized distilled water (ddH_2O). The quality and approximate yield was determined by electrophoresis of 5µl of genomic DNA mixed up with 1µl of 6× loading dye with 1kb DNA Ladder (GeneRuler™, Fermentas) on 1% agarose gel. The electrophoresis was run at 90 V for 45 minutes and visualized by using UV transilluminator.

3.3. Polymerase Chain Reaction (PCR)

PCR involves enzymatic amplification of DNA fragment by two flanking oligonucleotide primers hybridizing to opposite strands of the target sequence (Kolmodin and Birch, 2002). Universal primers (COIe and COIf) were used in PCR amplification and Table 1 shows its sequence.

Table 1: Primers for partial COI and its sequence (Palumbi, 1996).

Primer	Sequence
COIe (Reverse)	5'- CCAGAGATTGGGAATCAGTG-3'
COIf (Forward)	5'- CCTGCAGGAGGAGGAGAYCC -3'

Master mix for PCR was prepared in 1.5 mL eppendorf tube in the following order (Table 2).

was added. The mixture was then centrifuged at 13,000 rpm for 10 minutes. All liquid from the microcentrifuge tube was removed and the pellet was air dried. Lastly, the pellet was dissolved in 50µl of sterilized distilled water (ddH_2O). The quality and approximate yield was determined by electrophoresis of 5µl of genomic DNA mixed up with 1µl of 6× loading dye with 1kb DNA Ladder (GeneRuler^{TM}, Fermentas) on 1% agarose gel. The electrophoresis was run at 90 V for 45 minutes and visualized by using UV transilluminator.

3.3. Polymerase Chain Reaction (PCR)

PCR involves enzymatic amplification of DNA fragment by two flanking oligonucleotide primers hybridizing to opposite strands of the target sequence (Kolmodin and Birch, 2002). Universal primers (COIe and COIf) were used in PCR amplification and Table 1 shows its sequence.

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Primer	Sequence
COIe (Reverse)	5'- CCAGAGATTGGGAATCAGTG-3'
COIf (Forward)	5'- CCTGCAGGAGGAGGAGAYCC -3'

Master mix for PCR was prepared in 1.5 mL eppendorf tube in the following order (Table 2).

Table 2: Components of master mix.

Reagent	1 reaction (µL)
ddH_2O	15.8
10 X reaction buffer (Promega)	2.50
dNTP mix (10mM)(Promega)	0.50
Primer COIe (10mM)	1.25
Primer COIf (10mM)	1.25
$MgCl_2$ (Promega)	1.50
DNA template	2.00
Taq polymerase (5 units/µL)(Promega)	0.20
TOTAL	25.0

PCR amplification was performed in thermal cycler (Biometra T-Personal) under the following condition (Table 3).

Table 3: PCR profile

Step	Temperature (°C)	Time (min)	Cycle
Pre-denaturation	94	2	1
Denaturation	94	1	30
Annealing	52 - 56	1	
Extension	72	2	
Final extension	72	5	1
Soaking	4	∞	

3.4 Restriction Fragment Length Polymorphism (RFLP)

PCR product from each species was digested with ten restriction enzymes (*Csp6*I, *Alu*I from Fermentas and *Rsa*I, *Kpn*I, *Xho*I, *Sal*I, *BamH*I, *Hpa*II, *Pst*I, and *Hae*III from Promega) (refer to Table 4 for the recognition sites of each enzyme). The restriction digest containing 4µl of PCR product, 4µl of sterilized distilled water, 1µl buffer and 1µl restriction enzyme was incubated at 37°C for two to four hours. The DNA fragments was then separated on 3% agarose gel

containing ethidium bromide and visualized under UV light to check the digestion profiles, and photograph of DNA bands was taken on Polaroid film.

Table 4: List of restriction enzymes used and its recognition sites.

Restriction Enzymes	**Recognition Sites**
*BamH*I	G^GATCC
*Pst*I	CTGCA^G
*Sal*I	G^TCGAC
*Kpn*I	GGTAC^C
*Xho*I	C^TCGAG
*Rsa*I	GT^AC
*Alu*I	AG^CT
*Hpa*II	C^CGG
*Csp6*I	G^TAC
*Hae*III	GG^CC

^ cutting site

4.0 RESULTS

4.1 DNA extraction

Seventy-two samples from 25 species have been extracted. Some of the extraction products are shown in Figure 2. Smearing bands were observed in the extracted samples.

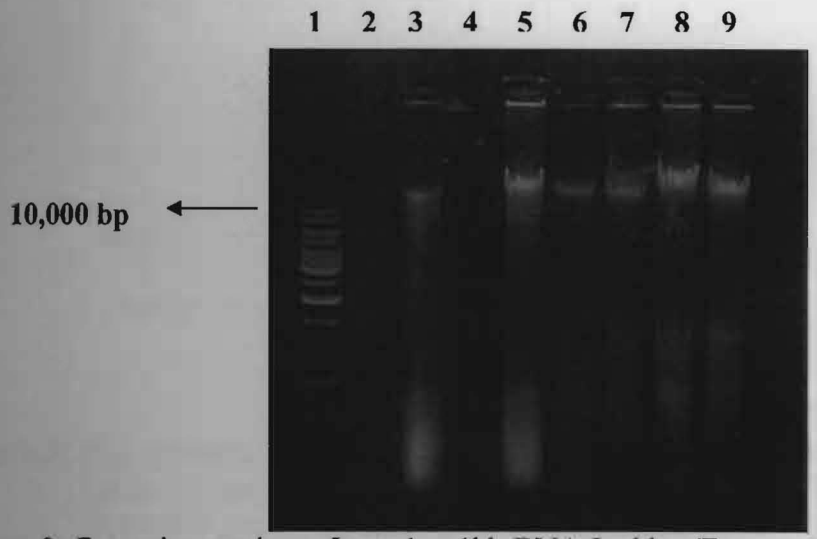

Figure 2: Extraction products. Lane 1 = 1kb DNA Ladder (Fermentas) as a standard size marker; Lane 2 = *Rhinolophus borneensis*; Lane 3 = *Hipposideros bicolor*; Lane 4 = *R. acuminatus*; Lane 5 = *R. luctus*; Lane 6 = *H. larvatus*; Lane 7 = *H. larvatus*; Lane 8 = *H. galeritus*; Lane 9 = *H. cervinus*.

4.2 Polymerase Chain Reaction (PCR)

Only samples that showed bright extraction bands were used for PCR amplification. PCR products produced in this study are approximately 500bp in length. Some of PCR products are shown in Figure 3. Primer-dimer was observed in some of the PCR products and there was no contamination occurred along the PCR amplification as there are no bands observed in the negative control lane (Figure 3).

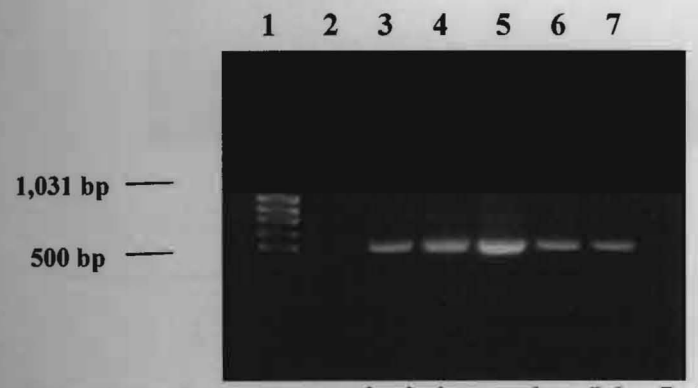

Figure 3: PCR products. Lane 1 = standard size marker (MassRuler™ Low Range DNA Ladder); Lane 2 = negative control; lanes 3-7 represents bright bands of PCR products (Lane 3 = *H. galeritus*; Lane 4 & 5 = *H. dyacorum*; Lane 6 & 7 = *H. cineraceus*)

4.3 Restriction Fragment Length Polymorphism (RFLP)

*Rsa*I (Figure 4 and 5), *Csp6*I (Figure 6), *Alu*I (Figure 9 and 10), *Hpa*II (Figure 7 and 8), *Sal*I and *Hae*III showed polymorphism between almost all species from the five families studied, however there were no restriction sites found with *Kpn*I, *BamH*I, *Xho*I and *Pst*I (results are not shown). There were also some species that were partly digested with *Rsa*I and *Hpa*II. During the